Deletions and missense mutations of EPM2A exacerbate unfolded protein response and apoptosis of neuronal cells induced by endoplasm reticulum stress

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The majority of the Lafora’s disease (LD) is caused by defect in the EPM2A gene, including missense and nonsense mutations and deletions. These defects mainly occur in the carbohydrate-binding domain, and how these mutations cause neuronal defects is under active investigation. Here, we report that the mutant proteins encoded by all missense mutations and most deletions tested are unstable, insoluble and ubiquitinated, and are accumulated in aggresome-like structures. The effect of apparent ‘gain-of-function’ mutations can be corrected by co-transfection of wild-type EPM2A cDNA, which is consistent with the recessive nature of these mutations in LD patients. In a neuronal cell line, these mutant aggregates exacerbate endoplasm reticulum (ER) stress and make the cells susceptible to the apoptosis induced by ER stressor, thapsigargin. The chemical chaperon, 4-phenylbutyrate, increased the mutant solubility, reduced the ER stress and dulled the sensitivity of mutant neuronal cells to apoptosis induced by thapsigargin and the mutant laforin proteins. The increased sensitivity to ER stress-induced apoptosis may contribute to LD pathogenesis.

INTRODUCTION

In Lafora’s disease (LD) patients, symptoms typically appear at the beginning of childhood and rapidly progress to severe myoclonic seizures, severe neurological deterioration, cognitive difficulty, dementia, muscle wasting and respiratory failure. Death usually occurs within 10 years of onset (1,2). So far, no prevention or cure is available to save the patient. Three typical manifestations of LD are progressive myoclonic epilepsy (PME), severe neurological deterioration and an accumulation of starch-like glycogen inclusion structures called Lafora bodies consist of polyglucosan. Lafora bodies are mainly found in neuronal perikarya and dendrites, liver, skin and muscle (2–4). Two genes with loss-of-function mutations have been identified in LD patients: EPM2A encoding a dual specificity phosphatase called laforin (5) and NHLRC1 encoding an E3 ligase named malin (6). Malin was revealed to be able to co-localize with and degrade laforin in the proteasome (5–8). Compared to those in NHLRC1, mutations in EPM2A gene are present in 80% of LD patients and produce more progressive courses of LD (9,10).

Laforin has been shown to be a phosphatase for GSK-3β, regulating both Wnt signaling and the cell cycle (11–14). Increased phosphorylation of GSK-3β was observed in mouse embryonic fibroblasts (11) and apparently in the brains of Epm2a−/− mice (15). Interestingly, the phosphatase activity for GSK-3β requires dimerization that is disrupted by tagging the protein at the N-terminus (12). In addition, laforin has been implicated in metabolism of glycogen (15,16). Moreover, laforin also confers cellular resistance to energy deprivation-induced apoptosis (17).

How the EPM2A and NHLRC1 mutations cause LD is under active investigation. Since LD is characterized by the formation of Lafora bodies containing insoluble and poorly
branched glycogen-like polysaccharides (18–20), the roles of the two proteins in glycogen metabolism have attracted a great deal of attention. The complex of laforin with malin inhibits glycogen accumulation in neuronal cells by down-regulating protein targeting to glycogen (PTG)-induced glycogen synthesis through a mechanism involving ubiquitination and degradation of PTG (21,22).

Analysis of the disease-causing mutations may offer insights into LD pathogenesis. So far, 18 missense mutations have been reported. This accounts for 42% of total mutations currently found in LD patients. These mutations are distributed in all four exons of laforin, and most of them occur in the two functional domains of laforin: carbohydrate-binding domain and dual specificity phosphatase domain (5,20,23).

We have shown that all of the seven naturally occurring mutations found throughout the EPM2A gene disrupt laforin dimerization (12). Since dimerization is required for its full phosphatase activity, our data showed a general mechanism for loss of function in the disease-causing mutations (12).

More recently, Dubey and Ganesh (24) showed that two mutations in the C-terminus (L310W and Q319 frameshift) abrogated heterodimerization of two isoforms of laforin, but not its homodimerization.

What is largely unclear is whether mutation laforin proteins may contribute to LD pathogenesis. Although mice with null mutation of laforin have some features of LD, they apparently have a normal lifespan (25). It is possible that in addition to the loss of function, mutations in LD patients may exacerbate neurological symptoms. In this regard, it is of interest that some laforin mutants were found to be aggregated and the aggregates appear to associate with proteasome (26). Existence of such aggregates suggests folding defects. Protein folding is a well-regulated process. Unfolded or misfolded proteins and peptides are often induced by genetic mutation, errors during transcription and translation, and by various stresses such as thermal, osmotic, energy deprived, endoplasm reticulum (ER) calcium depleted, heavy metal toxic or oxidative stresses. An accumulation of unfolded proteins can provoke ER stress. The unfolded or misfolded protein aggregates can affect or disrupt normal cell functions (27). Fortunately, ER stress can also induce coordinated adaptive machinery called unfolded protein response (UPR) that alleviates ER stress and avoids the destructive protein aggregation via the suppression of nascent protein synthesis, facilitation of protein folding and reinforcement of proteosome-mediated degradation of unfolded proteins (27). However, if the stress is beyond the capacity of UPR facilitation, the UPR can go in the opposite direction and induce cell apoptosis. Neurons are vulnerable to this ER-mediated UPR. Therefore, diverse protein aggregates are major pathological causes in neurodegenerative diseases such as familial Parkinson’s, Huntington’s and familial Alzheimer’s diseases (28,29).

Three ER transmembrane receptors of UPR relay signal pathways to control the ER stress. They are protein kinase R (PKR)-like ER kinase (PERK), activating transcription factor 6 and inositol-requiring enzyme 1 (27). In resting cells, all three receptors are maintained in the inactive state by their association with the ER chaperone, Glucose-related protein 78 (GRP78). Upon the accumulation of unfolded proteins, GRP78 dissociates from the three receptors. The dissociation of GRP78 activates the ER transmembrane receptors and triggers UPR. First, the dissociation of PERK from GRP78 initiates the dimerization and autophosphorylation of the kinase. The phosphorylated active PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to the inhibition of general protein translation and aiding cell survival by decreasing the load of nascent proteins in the ER in short term. However, a prolonged activation of the PERK-eIF2α signal pathway induces cell apoptosis (27). A chemical chaperone, 4-phenylbutyrate (4-PBA), has been demonstrated to reduce misfolding of several proteins (30) and ER stress in the mouse model of type 2 diabetes (31). Identification of the markers for UPR and the drugs that modulate UPR provided us with an opportunity to study potential dominant function of the laforin mutations in the pathogenesis of LD.

Here we report that the naturally occurring mutants tested, including all of 10 missense mutants and deletion mutants lacking of either exon-1- or exon-2-encoded amino acid sequences, formed aggregates and elicited low levels of ER stress. Importantly, these mutants sensitized neuronal cell to death induced by ER stressor thapsigargin that depletes Ca\(^{2+}\) from the ER via the inhibition of ER-associated Ca\(^{2+}\) ATPase. 4-PBA increased laforin mutant solubility and the amounts of protein, while preventing mutant aggregation and reducing ER stress. Our data provide a new insight on the pathogenesis and treatment of LD.

RESULTS

Laforin mutants form large ubiquinated aggregates in the cells

To analyze the potential function of the laforin mutants, we use site-directed mutagenesis to produce 10 mutations according to those found in LD patients, a phosphatase-dead mutation (C265S) was also introduced as control (Fig. 1A). When we transiently transfected equal amounts of plasmids encoding wild-type (Epm2a), phosphatase-dead mutant (C265S) and naturally occurring missense mutants into HEK293 (Fig. 1B) or NIH3T3 (Fig. 1C) cells, we observed less mutant proteins in the lysates when compared with WT proteins. Accumulation of proteins does not depend on phosphatase activity, as the C265S proteins were expressed to a similar extent in the wild-type (Fig. 1B and C). To determine whether the apparently rapid turnover of laforin occurred at the protein or at the RNA levels, we treated the WT or missense-mutant EPM2A-transfected cells with either the inhibitor for protein synthesis, cycloheximide (Fig. 2A), or the inhibitor of the RNA synthesis, actinomycin D. We measured the amounts of monomeric forms of laforin after boiling in the presence of SDS (Fig. 2B). After normalizing against protein loading, the ratios of treated over untreated groups were listed underneath the immunoblots. Our data demonstrated that while protein synthesis inhibitor did not affect the amounts of WT laforin, it decreased the amount of missense mutants by 1.7–3.5-fold. In contrast, actinomycin D did not significantly change the amounts of missense mutant protein. Therefore, the levels of the mutants were regulated by turnover at the protein level rather than mRNA level.

At least two mechanisms can potentially explain the reduction of steady-state protein levels: degradation by
proteasome and/or formation of detergent-insoluble aggregates. We first treated the transfectants of two missense mutants, F83L and Q292L, with an effective dose of proteasome inhibitor, MG132. As shown in Figure 3A, MG132 barely increased protein amount of these mutants. This indicated that proteasome-mediated degradation is not the main factor that caused the observed disappearance of these mutants. We then analyzed the solubility of mutants by comparing the amounts of laforin in the supernatants or the pellets of cell lysates. Based on the ratios of the proteins in the two fractions, it is clear that 75% WT and 85% C265S proteins appeared in the soluble fraction, while usually well below 50% of missense mutants are soluble (Fig. 3B). Using a higher concentration of Triton X-100, some large aggregates can be found in the soluble fraction. This provided us with an alternative approach to compare the amounts of monomer/dimer versus the aggregates. Using immunoprecipitation (IP) followed by a western blot, we recapitulated the substantial increase in the amounts of aggregated laforin in mutant EPM2A-transfected cells based on their size (Fig. 3C). Thus, for WT and C265S mutants, the amounts of monomers and dimers are about six times as many as that of the aggregates. In contrast, the majority of the F88L and Q293L mutants appeared as aggregates. Even though more W32D appeared as monomer, about one-third of the proteins appeared as aggregates. Importantly, deletion of all or part of exon-1 as well as all of exon-2-encoded sequence also caused formation of large aggregates, with less than 30% appeared as monomers. Interestingly, deletion of exon-3-encoded sequence significantly increased the amounts of aggregates (Fig. 3C).

Furthermore, analysis of the ubiquitination status of these mutants indicated that these mutants were polyubiquitinated in the absence of MG132. As shown in Figure 4A, anti-polyubiquitin reacted with high molecular weight species that partially overlapped with anti-V5 antibody. In addition, anti-V5 also recognized non-ubiquinated monomer and dimer of laforin. To substantiate ubiquitination of laforin, we co-transfected ubiquitin-HA cDNA in conjunction with cDNAs encoding either Myc- or V5-tagged laforin. The lysates were immunoprecipitated with either anti-Myc or anti-V5 mAb and probed with both anti-laforin and anti-HA. As shown in Figure 4B top panel, missense mutations reduced the amounts of both monomeric and dimeric form of laforin. The extent or reduction varied somewhat among...
Figure 2. Reduction in mutant proteins occurs on protein rather than mRNA levels. HEK293 cells were transiently transfected with WT or mutant laforin. After 24 h, they were treated with 50 μM Cycloheximide for 2 h (A), or with 2.5 μM Actinimycin D for 8 h (B). Laforin proteins in 1% Triton-X-100 lysate were detected by anti-V5 immunoblotting. The S6K bands act as loading control. The density ratios of non-treated to treated laforin bands after subtracting the density of control S6K band was shown at the bottom of panels. Asterisk indicates babble in the transfer process that has caused less protein of S6K. The ratios of the amounts of protein in untreated over treated samples are listed underneath the graphs.

Figure 3. The apparent reduction of the mutant protein is due to aggregation rather than proteasome-dependent degradation. (A) Proteasome degradation is not responsible for rapid turnover. HEK293 cells were transiently transfected with WT or mutant laforin. After 24 h, they were treated with 2.5 μM MG132 for 16 h for the analysis of the amounts of soluble laforin mutants, as revealed by western blot with anti-V5. The amounts of β-actin were used as loading control. (B) Missense mutations of EPM2A reduce solubility of laforin. After a 24 h transfection, the transient transfectants of HEK293 cells were lysed by 0.5% Triton-X-100. The lysates were spun at 16,100 g for 10 min to collect both supernatants (Triton-soluble) and pellets (Triton-insoluble) for western-blot analysis using anti-V5 for laforin or anti-S6K for loading control. Equal aliquots of soluble and insoluble fractions were boiled in 2% SDS prior to electrophoresis. The ratios of soluble over insoluble forms are provided in the bottom of lower panel. (C) Both deletional and missense mutants of laforin form insoluble aggregates. The HEK293 cells were transfected with WT, missense and deletional mutants. At 48 h after transfection, the cells were lysed with 1% Triton X-100. The lysates were spun at 16,100g for 10 min to collect supernatants for standard IP and western blot with anti-V5 mAb. The ratio of dimer+monomer over aggregates was listed underneath the photograph.
different mutants, with the least reduction observed in W32D and R108C mutants. Reciprocally, anti-HA mAb detected a massive increase polyubiquinated laforin in all missense mutants tested. Again, the increases were more pronounced among those mutant that show the least amount of monomers and dimers. These findings demonstrated that missense mutants of laforin formed ubiquitin-positive aggregate that cannot be completely degraded by proteasome.

**Laforin mutants are ubiquinated and co-localized with aggresome**

We stained transiently transfected HEK293 cells (Fig. 5A and B) or neuronal Neuro2a cells (Fig. 5C) with anti-V5 mAb. Our data demonstrated that these mutants form small clots or large aggregates around the nuclei in both cell types. In contrast, wild-type laforin protein localized throughout the cytosol (Fig. 5A and C). The percentage of V5+ cells with V5+ aggregates was up to 12–35% among the natural mutant transfecteds, while the number of aggregates of WT or the C265S mutants was barely detectable (Fig. 5B).

We further analyzed the mutant aggregates in Neuro2a by using antibodies against common markers of aggresomes, including 20S proteasome, intermediate filament vimentin and ubiquitin. We found that aggregates overlapped with all three markers, which is consistent with localization into aggresome. One representative of F83L is shown in Figure 5C. These results are consistent with the ubiquitination of mutants detected by IP-Western blot (Fig. 4) and the results of missense mutant Q293L in human neuroblastoma cell line SH-SY5Y reported by Mittal et al. (26).

**Naturally occurring laforin mutants elicited unfolded protein response (UPR) and sensitizes cells to thapsigargin-induced apoptosis**

Cells that cannot efficiently eliminate aggregates of unfolded proteins will provoke ER stress and activate the UPR. The UPR can be manifested by an increase in a group of molecular chaperons or co-chaperons in the ER such as GRP78 and HSP70 (32). In COS-7 or neuronal Neuro2a cells transiently transfected with wild-type or mutant laforin, we found that mutant F83L slightly increased the protein expression of chaperon GRP78 in COS-7 cells (Fig. 6A) and Neuro2a cells (Fig. 6B), in the absence of ER stressor. When provoked by ER stressors, such as, MG132 (proteasome inhibitor), tunicamycin (disruption of glycosylation of newly synthesized proteins) or thapsigargin (depletion of Ca2+ in ER), F83L dramatically induced HSP70 and/or GRP78 (Fig.6A and B) and activated eIF2α, a major component of ER stress signal pathways (Fig. 6B).

We established a Neuro2a cell line that expressed F88L mutant under the tetracycline-off control. Upon exposure to thapsigargin, the mutant activated Caspase 3 (Fig.7A). Transient transfection of laforin mutant into the Neuro2a also increased the cell susceptibility to thapsigargin-induced death (Fig. 7B).

**Chemical chaperon 4-PBA increases solubility of mutants and decrease mutant-elicited ER stress and death**

It is known that the chemical chaperon, 4-PBA, increase stability and solubility of several mutant proteins by facilitating
protein folding and alleviating proteasome-mediated degradation (30,31). To test whether 4-PBA could improve the stability and solubility of laforin mutants, Neuro2a cells were transiently transfected with different mutants and then incubated with lysis buffer containing 1% Triton-X-100. Compared to the control vehicle, 4-PBA significantly increased the protein amount of mutants (Fig. 8A, top pair panel). In contrast, 4-PBA had no effect on WT laforin. Consistent with the increase of soluble mutant proteins, 4-PBA reduced phosphorylation of eIF2α at 4 h after the mutant cells were exposed to trapsigargin (Fig. 8A, low pair panel). Furthermore, 4-PBA reduced the death of mutant F83L cells 24 h after trapsigargin treatment (Fig. 8B).

**WT laforin prevents mutant aggregations and alleviates mutant’s impact on ER stress**

Since heterozygous patients are asymptomatic, we tested if the WT laforin inhibits aggregation of the mutant proteins. We co-transfected either control vector or WT Epm2a cDNA with five given missense mutants into Neuro2a cells and compared the amounts of aggregated mutant proteins. Our data demonstrated that WT laforin reduced the mutant aggregates, particularly the larger aggregates (Fig. 9A). By IP and western-blot analysis, we showed that all but one (P300L) mutant formed dimers with WT laforin, albeit much less efficiently than homodimerization of WT laforin (Fig. 9B).

To test the effect of WT laforin in the laforin mutant-exacerbated ER stress response, we co-transfected WT laforin or its phosphatase-dead mutant C265S with mutant F83L into the neuronal cells and used the amounts of GRP78 as an indicator of ER stress response. As shown in Figure 9C, WT laforin reduced the GRP78 amounts, which suggest that laforin mutant exacerbated response can be alleviated by WT laforin. Interestingly, the C265S mutant enhanced the mutant-predisposed ER stress regardless of the second ER stress hit (Fig. 9C). Therefore, the protective effect of laforin depends on its phosphatase activity. The exacerbation by the phosphatase-dead mutant indicated that this mutant may work as a dominant negative for the endogenous laforin function which is in agreement with the phenotype of the transgenic mice expressing the phosphatase-dead mutant (33).

**DISCUSSION**

Despite the identification of causative mutations, the pathogenesis of LD remains largely unexplained. Since the Lafora body, a diagnostic marker of LD, consists of starch-like
glycogen polyglucosan (3,4), the involvement of glycogen metabolic defects in LD has attracted a great deal of attention (21,34). However, since patients with glycogen storage disease usually do not have PME (35), defects in the glycogen metabolism may be insufficient to explain the pathogenesis of LD.

Previous studies indicated that five out of seven laforin mutants tested formed large aggregates within the transfected cells (7,26,36). We have recently reported that laforin mutants dramatically impaired dimerizations, indicating that mutants could be abnormally folded (12). Here we demonstrated that this is likely the case as all of the 10 missense mutant proteins tested in this paper, including two that were reported to be soluble by others (37) formed insoluble and/or polyubiquinated aggregates. The apparent difference between our studies and that by Ganesh et al. (37) remains to be reconciled. These two mutants have mutations near the N-terminus. We found that tagged N-terminus in laforin may affect dimerization (12). Either a difference in the strategy in tagged proteins or stress levels of cultivating condition might contribute to the difference. In combination, all 15 laforin missense mutants have been demonstrated to form aggregates in some assays. Therefore, this is likely to be a general feature of the missense mutants. Moreover, we demonstrated that deletion of exon-1- or exon-2-encoded sequence, which has been reported in LD patients (10,37), also caused aggregation of the laforin. Unfortunately, due to lack of reagents, the chemical analysis on laforin protein in the LD patients remains relatively scarce.

Interestingly, the misfolded mutant proteins render neuronal cells more susceptible to apoptosis induced by extra ER stress. Moreover, chemical chaperon 4-PBA increased mutant solubility and reduced cell death exacerbated by mutants. These data raised the possibility that in addition to functional inactivation of the physiological function of EPM2A, the mutant protein may play an active role in the pathogenesis of LD.

Analysis of mice with targeted mutation of Epm2a has revealed that the Epm2a gene is critical for neuronal cell survival, as null mouse’ neuronal cells died from the swelling...
of ER and damaged mitochondria and Golgi networks (25). Nevertheless, the Epm2a-null mouse can live for its full life-span, whereas LD patients usually die within 10 years of disease onset. One way to reconcile this apparent inconsistency is the dominant role of the misfolded mutant aggregates in the pathogenesis of LD, together with recessive defects of EPM2A, leads to full-spectrum of LD observed in human patients with bi-allelic inactivation.

The notion of ‘gain-of-function’ mutation appears incompatible with the clinical observation that heterozygous individuals are asymptomatic. However, one of the primary functions of laforin and malin is degradation of aggregated proteins (36). Therefore, a functional laforin–malin complex in the heterozygotes should be able to remove the mutant aggregates of laforin, hence explaining the recessive phenotype for the gain-of-function mutations. While the mechanism proposed herein is by no means mutually exclusive with others, our hypothesis has two useful features. First, it may unite the pathogenesis of PME caused by genes other than EPM2A and NHLRC1. Second, it suggests that targeting ER stress pathway can be explored for potential therapeutic approach.

MATERIALS AND METHODS

Antibodies and compounds

Mouse monoclonal antibodies of V5 and Myc (Invitrogen), HA (Roche), Ubiquitin (P4D1, Santa Cruz), HSP70 (Santa Cruz), GRP78/Bip (BD Transduction Lab), S6K (H-9, Santa Cruz), β-actin (Sigma) and rabbit antibodies of cleaved Caspase 3 (Cell Signaling), 20S proteosome (Biomol), ubiquitin (FL-76, Santa Cruz) for immunofluorescence staining, and laforin (produced by Genemed Synthesis, Inc., San Francisco, CA, USA), goat anti-vimentin (Millipore) were used in the present study. Tunicamycin, thapsigargin, 4-PBA and actinomycin D were purchased from Sigma. The MG132 and protein G beads are from Calbiochem and Amersham, respectively.

Plasmids

All expression plasmids of V5 or Myc tagged on the C terminus of Epm2a, and its deletional and missense mutants are constructed as described previously (12). All mutants made by site-directed mutagenesis using the template of wild-type Epm2a-V5 were sequenced for confirmation. TRE-F88L-V5 construct was made by cloning human mutant F88L into TRE-Myc vector (Clontech), and then transferring the TRE-F88L fragment into pLenti6/TO/V5 vector (Invitrogen) after removing the CMV promoter in the plenti vector.

Tansfection and lenti-virus preparation

Generally, human embryonic kidney HEK293 cells were transiently transfected or cotransfected with total 1 μg DNA/well on six-well plate of different expressing plasmids for 24–36 h using O-piti medium containing 10% fetal bovine serum and lipofectman-2000 (Invitrogen). The transfected cells were then lysed with a regular lysis buffer containing 0.5 or 1% Triton-X100. TRE-F88L lenti-virus was made according to the lenti-virus instruction of Invitrogen.

Western blot and immunoprecipitation (IP)

Transfected HEK293 cells were lysed by 1 or 0.5% Triton-X100 lysis buffer containing each of protein inhibitor cocktail and phosphatase inhibitor cocktail (Sigma) at 1:100 dilution. Supernatants of the lystate were used for western blot. For IP, 1% Triton-X100 lysate supernatants after pre-rotated with protein G beads for 1 h were used at constant rotation for overnight at 4°C. The protein G beads captured the IP complex again by an additional 2 h rotation at 4°C. Following three washes with 1 ml of 1% Triton-X100 lysis buffer, the IP complex was dissociated from protein G beads by SDS loading buffer and run a 10% SDS–PAGE.
Immunofluorescence
At 24 h after transfection, the HEK293 and Neuro2a cells were treated with vehicle DMSO or 2 mM of thapsigargin for 4 h and fixed by methanol. The fixed cells were permeabilized with 0.3% Triton X100, stained with primary antibodies of anti-mouse V5, anti-rabbit ubiquitin or anti-rabbit 20S. The primary antibodies were detected with second antibodies of anti-mouse Texas red and anti-rabbit fluorescence.

MTT assay
Transiently transfected Neuro2a cells in triplicate wells of 24-well plate were treated with DMSO vehicle, different concentrations of thapsigargin in conjunction with or without 1 mM 4-PBA. The amounts of viable cells were quantitated by adding 5 μM MTT dye and incubated in 37°C incubator for 4 h. The absorbed dyes were collected by centrifugation and dissolved in DMSO. The optical absorbency was monitored at 490 nm.

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